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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b>  Increased fibrosis and integrins expression are elevated in in APDKD. The scope of the study is to assess whether integrin beta1 (Intβ1) plays a role in the ADPKD. The past funding period has focused primarily on the in vivo study of the role of in the cystogenic process. We have generated conditional double knockout mice where both Itgb1 and Pkd1 genes can be simultaneously deleted. As previously described, the single conditional Pkd1 knockout develops an overt cystic phenotype by 4 weeks, whereas the conditional deletion of Itgb1 had no observable effects. Interestingly, the simultaneous ablation of Itgb1 in compound Pkd1/Itgb1 knockout mice significantly prevented renal cystic development. Correspondingly, while the renal function of the Pkd1 knockout mice was significantly declined in time, it remained unaffected in the double knockout mice for all the time points so far tested. Although the studies are still ongoing, these findings support the initial hypothesis that Intβ1 is an essential mediator in ADPKD cystogenesis. These results indicate that the signaling pathway controlled by Intβ1 could be successfully targeted to prevent or slow down the cystic development.					
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## INTRODUCTION

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common monogenic disorder. The disease is characterized by progressive renal tubules dilatation, bilateral formation and expansion of multiple septated fluid-filled cysts that increasingly compress the renal parenchyma thus altering the kidney architecture and gradually impairing organ function. To date there is no treatment for ADPKD, which remains the fourth leading cause of end stage renal disease.

The progress in developing an efficacious therapy for ADPKD is hampered by the complexity of the cystogenic mechanisms that remain incompletely understood.

A hallmark of ADPKD is an increase in interstitial fibrosis, which parallels the progression end stage renal disease<sup>1</sup>. The abnormal deposition of extracellular matrix may induce aberrant cellular responses that promote the cystic processes. The evidence that some integrins that function as receptors for ECM components are overexpressed in cystic epithelia supports the possibility that abnormal matrix interactions affect proliferation and cell differentiation<sup>2</sup>. We previously showed an increase of integrin- $\alpha 2\beta 1$  expression in polycystin-1 (PC1) deficient cells, which was important in the cell survival<sup>3</sup>.

Based on these observations, we formulated the hypothesis that  $\text{Int}\beta 1$  is an important component of the cystogenic mechanism ADPKD1. The objective of the study was to dissect the role of  $\text{Int}\beta 1$  both *in vitro* on the proliferation and centrosome integrity and *in vivo* on the cystic process.

In the previous report we presented the results from our *in vitro* approach with the findings that functional changes that are specific to PC1 deficient cells, such as their proliferative phenotype and centrosome aberrations, could be reverted to normal control levels by the knockdown of  $\text{Int}\beta 1$ . In the past year we have focused our efforts on the *in vivo* validation of the role of  $\text{Int}\beta 1$  on the cystic process. Our results corroborate the *in vitro* observations and indicate that  $\text{Int}\beta 1$  is an essential mediator of cystogenesis triggered by the loss of PC1.

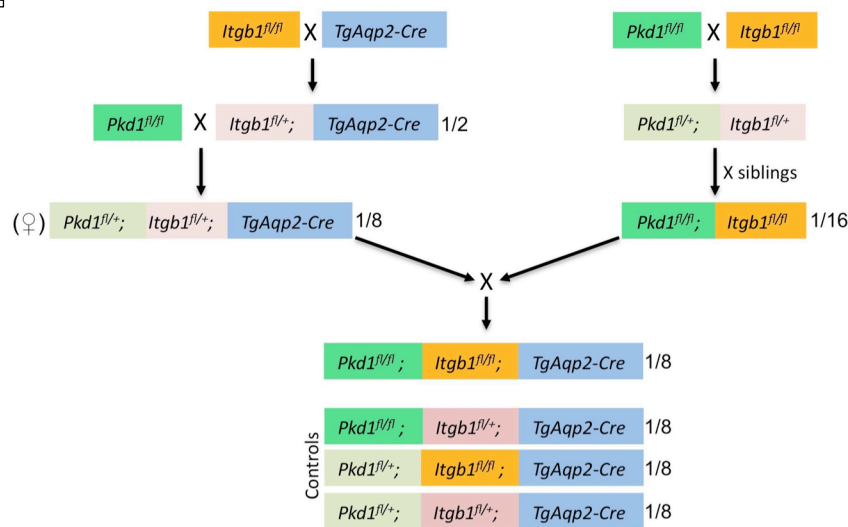
## BODY

**Reversal of cystic phenotype in the absence of  $\text{Int}\beta 1$ .** In the last report we illustrated our strategy to determine the *in vivo* role of  $\text{Int}\beta 1$  based on the crossing of  $Pkd1^{fl/fl}$ ;  $Itgb1^{fl/fl}$  double floxed mice with the  $TgAqp2\text{-cre}$  mice in which the expression of Cre is under the control of the aquaporin-2 (Aqp2) promoter. This promoter is activated at late developmental stages (E18.5) specifically in the renal collecting ducts<sup>4</sup>. The expression of Cre at this late stage of development circumvents the embryonic lethality associated with the constitutive knockout of either gene (the breeding scheme and the resulting genotypes and relative frequencies is shown in Figure 1).

During the past funding period, we crossed these strains to obtain a homozygous  $Pkd1^{fl/fl}$ ;  $Itgb1^{fl/fl}$  double floxed mouse and a heterozygous  $Pkd1^{fl/+}$ ;  $Itgb1^{fl/+}$ ;  $TgAqp2\text{-cre}$ . These mice were subsequently crossed to produce the experimental single  $Pkd1^{fl/fl}$ ;  $TgAqp2\text{-cre}$  and the double knockout  $Pkd1^{fl/fl}$ ;  $Itgb1^{fl/fl}$ ;  $TgAqp2\text{-cre}$  mice.

Gestation rates and litter sizes were unremarkable in comparison to control wild-type mice, and genotype frequencies did not vary significantly from the expected Mendelian distribution, confirming the lack of embryonal lethality. However, by 4 weeks (P28) it was evident that despite comparable thriving conditions some of the animals with the single  $Pkd1$  deletion ( $Pkd1^{fl/fl}$ ;  $Itgb1^{fl/+}$ ;  $TgAqp2\text{-cre}$ )

□



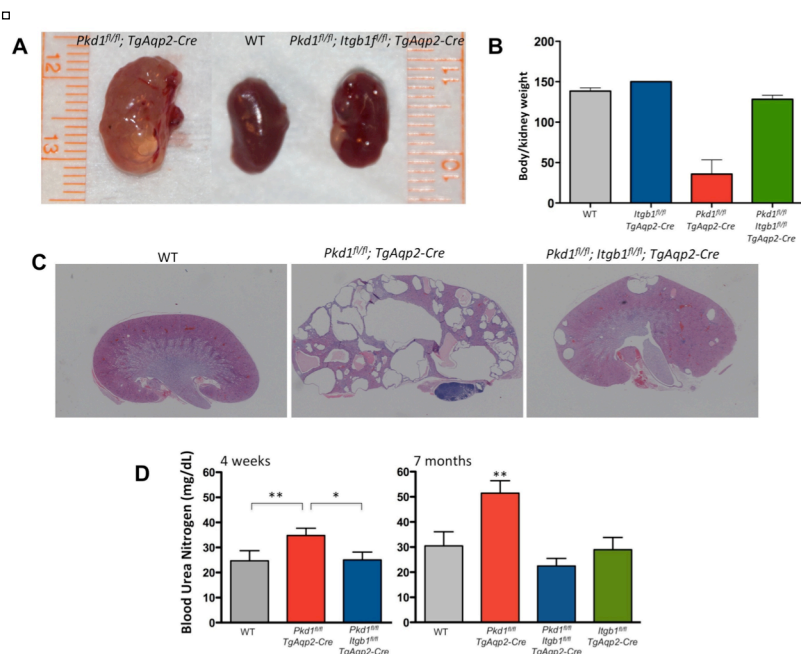
**Figure 1.** Breeding scheme for the generation of the double floxed  $Pkd1^{fl/fl}; Itgb1^{fl/fl}$  mouse. Note that since the Cre activity in  $TgAqp2-Cre$  transgenic mouse was observed also in testes, our breeding scheme was devised to ensure maternal inheritance of the transgene, as paternal transmission would lead to gene recombination not only in the kidneys, but also in sperm and the resulting fertilized oocytes. Numbers on the side indicate the relative frequency of the genotype.

were of smaller size, with body weight significantly lower than littermates (not shown). Kidneys from 6-week old mice were grossly enlarged and histological analysis established the advanced degree of cystic disease extended to the whole organ (Fig. 2A), confirming what was expected from the early inactivation of the  $Pkd1$  gene<sup>5</sup>. In stark contrast, the kidneys from mice in which  $Itgb1$  was inactivated simultaneously to  $Pkd1$  ( $Pkd1^{fl/fl}; Itgb1^{fl/fl}; TgAqp2-cre$ ) were not only normal in size but with an histological appearance closer to the kidneys of wild-type rather than the single  $Pkd1$  ablated mouse (Fig. 2).

Although focal cysts were present, these were far fewer and smaller in size with limited distribution in the cortical area indicating a significantly milder cystic phenotype. Despite the

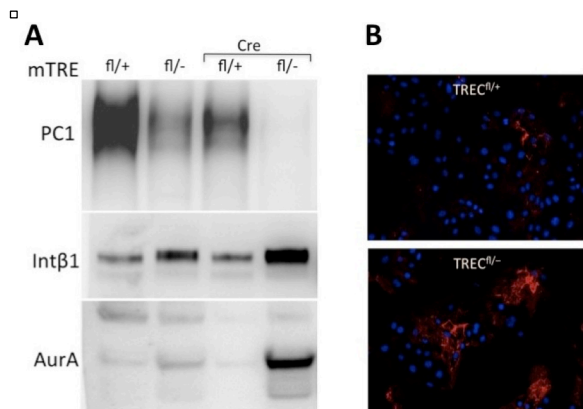
localization of the focal cysts, positive staining with *Dolichos bifluoros* agglutinin (DBA) confirmed their collecting duct origin (not shown). Body to kidney weight ratio in the single  $Pkd1$  knockout was significantly reduced as compared to the compound  $Pkd1; Itgb1$  knockout, which was comparable to the wild type and single  $Itgb1$  knockout. Histologically, the kidneys of the double heterozygous  $Pkd1^{fl/+}; Itgb1^{fl/+}; TgAqp2-cre$  and single  $Itgb1$  deletion ( $Pkd1^{fl/+}; Itgb1^{fl/fl}; TgAqp2-cre$ ) mice were indistinguishable from those of the wild-type as expected in agreement with the published literature<sup>6</sup>. Correspondingly, the levels of blood urea nitrogen showed that the renal function was significantly altered in the animals with renal  $Pkd1$  ablation but normal in the double knockout mice (Fig. 2D).

Note that since the  $Pkd1$  floxed allele and the  $Itgb1$  floxed allele are in a C57BL/6 background and 129Sv/J, respectively, the double floxed animals possess a mixed genetic background. The mixed background affects the susceptibility of the animals to the cystic disease, allowing the animals to live beyond the average 8-week life expectancy described in the C57BL/6 background<sup>7</sup>. This characteristic is captured by the data on BUN in figure 2D, which show a slow but progressive decline of the renal function. In this regard, this model is more representative of the course of the human disease and will be more sensitive for the study of anti-cystic treatments. However, longer time is required to determine the effects of the double knockout on survival. This characteristic and the relatively low frequency of each compound genotype have expanded the time required for the completion of the analysis of disease progression and survival that we plan to complete within the next year.



**Figure 2. Ablation of *Intβ1* in vivo prevents overt cystogenesis caused by the absence of PC1.** (A) Whole kidneys from conditional single *Pkd1* and double *Pkd1;Itgb1* knockout compared to WT. (B) Average ratio of body/kidney weight from the different genotypes. (C) H&E staining of sagittal sections of representative kidneys from control WT, single and double knockout animals. As described, kidneys from the single conditional *Itgb1;Tg-Aqp2-Cre* mice present no significant findings. All data from 6-week old mice (A, B, C). (D) Values of blood urea nitrogen from WT, single and double knockout mice at indicated ages. \* $P < 0.05$ ; \*\* $P < 0.01$ .

**Generation of floxed *Pkd1* cell lines.** To establish an *in vitro* system for the conditional ablation of *Pkd1* gene, we isolated renal epithelial cells from kidney papillae of *Pkd1<sup>fl/+</sup>* and *Pkd1<sup>fl/-</sup>* genotypes obtained following the crossing of the previously described *Pkd1<sup>+/-null</sup>* (kindly provided by Dr. Jing Zhou) and floxed *Pkd1<sup>fl/fl</sup>* (*B6.129S4-Pkd1<sup>tm2Ggg</sup>/J*, Jackson Laboratory) mouse models<sup>5,6</sup>.



**Figure 3. Ablation of *Pkd1* in floxed *Pkd1* mTert-immortalized cell lines.** (A) The loss of PC1 increases the expression of *Intβ1* and *AurA*. (B) Higher fibronectin (red) deposition is detectable after the deletion of *Pkd1* in mTREC<sup>fl/-</sup> cells. Nuclei were counterstained with DAPI (blue).

We immortalized these cells similarly to what was described by Steele et al.<sup>8</sup>, using a lentivector expressing the murine telomerase reverse transcriptase (mTert). After few passages, these cells were cultured for 24 hours in high osmolarity medium (700mOsm/kg) to enrich for collecting duct cells. Their epithelial nature was confirmed by cytokeratin staining and by the high transepithelial resistance on transwell membrane (>1000 Ohm/cm<sup>2</sup>) (not shown). Expression of *Aqp2* and absence of *uromodulin* confirmed the collecting duct origin of the cells, which will be hereafter referred to as mTREC<sup>fl/+</sup> and mTREC<sup>fl/-</sup> (Fig. 3A). (A similar approach based on mTert has been utilized for the immortalization of cell lines from wild-type and double floxed mice. The isolation of clonal populations is underway.)

Following Cre expression mediated by lentivector that confers resistance to blasticidin, the expression of PC1 was entirely abrogated in the mTREC<sup>fl/-</sup> cells (mTREC<sup>fl/-</sup>)

(Fig. 3A). This coincided with the expected increase in *Intβ1* expression as well as *AurA*, a mitotic kinase involved in centrosome integrity, spindle stabilization, control of cilium reabsorption, and calcium signaling<sup>9-11</sup>. Interestingly these changes were paralleled by the increased deposition of fibronectin in the *Pkd1* ablated cells (Fig. 3B).

## KEY RESEARCH ACCOMPLISHMENTS

- Double conditional knockout mice have been generated
- *In vivo* experiments indicate that  $\text{Int}\beta$  is required for the cystic development.
- Cell lines from murine collecting ducts have been generated in which *Pkd1* can be conditionally inactivated. This will be a useful model to investigate the immediate effects of PC1 inhibition (e.g. centrosome amplification and genomic instability) on cellular functions.

## REPORTABLE OUTCOMES

- A manuscript describing the findings is in preparation and will be finalized as soon as the experiments on the progression of ESRD and survival will be completed.
- Data from this work have been used for a R01 grant application to the NIDDK.

## CONCLUSIONS

Although the study is ongoing, the current findings confirm our initial hypothesis that  $\text{Int}\beta 1$  is required for the cystogenic process of ADPKD. The future experiments will conclude the analysis on the survival and allow us to further characterize the extent of protection with regard to other parameters that are associated with the cystic process such as fibrosis and apoptosis. Importantly, these results have revealed a previously unrecognized signaling pathway essential for cystogenesis that could be targeted therapeutically.

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## **APPENDICES**

None